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(54) Title: METHOD OF CELL CULTURE

(57) Abstract

The present invention relates to a method of culturing a mixed population of neuronal and glial cells which method includes (i) providing a tissue sample which includes a heterogeneous population of neuronal and glial cells; (ii) dissociating the neuronal and glial cells by enzymatic digestion; (iii) separating the dissociated neuronal and glial cells from aggregate cells; (iv) plating the dissociated cells on a support means until the cells have attached thereto and grown to a desired concentration; and (v) maintaining the cell population attached to the support by addition of serum-free media.

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*Method of cell culture*

FIELD OF THE INVENTION

The present invention relates to a method of culturing a primary mixed population of neuronal and glial cells. The present invention also relates to methods of screening neuronal growth factors, neuroprotective agents, neurotoxins, therapeutic or prophylactic agents and agents that affect the activity of cells.

BACKGROUND OF THE INVENTION

Primary cultures of neurons have many advantages over established immortalised cell lines in terms of providing model systems for studying neurogenesis and cell death. These advantages include the ability to maintain a reasonably true phenotype and the ability to undergo neurogenesis and senescence in culture. Mixed primary cultures of neuronal and glial cells provide even further advantages. These heterogeneous cell populations more accurately reflect the normal tissue of origin and therefore allow studies of processes which parallel normal behaviour. Heterogeneous cell cultures also allow expression of receptors and growth factors which may not be homogeneous throughout the tissue. The heterogeneity of the cells, both in cell type and stage of development, is therefore a further advantage in model cell cultures.

Primary cultures of nervous system cells may be used to study the effects of various growth factors. In order to perform these studies, however, it is important that the cell cultures are free from contaminating growth factors. Cell culture systems which involve nutrient media containing supplements such as serum are unsuitable for this purpose as serum samples usually contain a variety of growth factors which cannot be eliminated or controlled. Accordingly, cell culture systems which can be maintained in serum-free media are also desirable.

Previous attempts to grow olfactory neuroepithelial cell cultures have primarily focused on attempting to grow pure cultures of neurons or alternatively pure cultures of glial cells *[Ronnet, G.V., Hester, L.D., and Snyder, S.H. (1991) Primary culture of neonatal rat olfactory neurons. J Neurosc. 11:1243-55; and Pixley, S.K. (1992) The olfactory nerve contains two populations of glia identified both in vivo and in vitro. Glia 5: 269-84]* The first of these methods by *Ronnett et al (1991)* was dependent upon adding exogenous fetal calf serum and NGF to the culture media and both of these

additions negate the value of these cultures in terms of studies related to growth and responsivity to growth factors .

Additionally, the second method by *Pixley (1992)* describes serum-free culture conditions for glial cells but these were pure glial cultures rather than heterogenous populations of neuronal and glial cells. The same investigator has published a method of short-term serum-free cultures of olfactory neurons *[Grill, R. And S. Pixely, (1993) 2-Mercaptoethanol is a survival factor for olfactory, cortical and hippocampal neurons in short-term dissociated cell culture. Brain Research, 613: 168-172]* but again this method does not define mixed neuronal and glial cell cultures. Further, these cultures were only maintained for 4 days. The long term viability of neuroepithelial cell cultures is essential for studies of growth factors, cell differentiation and longevity.

Other workers have successfully cultured olfactory neurons from rats, but have plated them out onto monlayers of ensheathing cells or cortical astrocytes *[Chuah, M. I. and Au, C. (1994) Olfactory cell cultures on ensheathing cell monolayers. Chemical Senses 19: 25-34]* which provide trophic support. These support cells, however, are a source of contaminating growth factors which limits the utility of the culture system.

SUMMARY OF THE INVENTION

The present inventor has now devised a cell culture method which provides primary cultures of heterogeneous neuronal and glial cell populations which have relatively long term viability (ie. the cells survive for up to three weeks in culture and then senesce) and which may be maintained in a serum-free environment.

Accordingly, in a first aspect the present invention provides a method of culturing a mixed population of neuronal and glial cells which method includes

(i) providing a tissue sample which includes a heterogeneous population of neuronal and glial cells;

(ii) dissociating the neuronal and glial cells by enzymatic digestion;

(iii) separating the dissociated neuronal and glial cells from aggregate cells;

(iv) plating the dissociated cells on a support means until the cells have attached thereto and grown to a desired concentration; and

(v) maintaining the cell population attached to the support by addition of serum-free media.

In a preferred embodiment of the invention the tissue sample is olfactory neuroepithelial tissue.

The tissue sample may be derived from a variety of animal sources. For example, the tissue sample may be derived from a chicken, rat or frog. Preferred animal sources are mammals such as rats, mice, primates and humans. In a preferred embodiment, the tissue sample is derived from a rat. Preferably, the tissue is derived from a neonatal rat.

The tissue sample may be obtained by dissection as is known in the art.

In a preferred embodiment the tissue is minced to provide small pieces of tissue prior to the step of enzymatic dissociation. The small pieces of tissue may be of sizes of approximately 1 $mm^2$.

In a further preferred embodiment the enzymatic dissociation involves the use of one of more enzymes selected from the group consisting of dispase, collagenase, hyaluronidase and DNAse. Typically these enzymes are incubated with the tissue at 37°C to digest the substances which may bind and aggregate the cells to each other.

In a further preferred embodiment the dissociation of neuronal and glial cells further includes mechanical disruption. The mechanical disruption may include any means known in the art, such as trituration, Waring blender, Potter-Elvenhjem homogenizer, meat grinders, shakers etc. Preferably, the mechanical disruption involves trituration.

In a further preferred embodiment the separation of the dissociated cells involves a size fractionation procedure. The size fractionation procedure may involve selective filtering of neuronal and glial cells. Preferably, the filtering involves passing the cells through a first filter which has a pore size of from 40 to 60 μm and through a second filter which has a pore size of from 8 to 15 μm. Preferably, the first filter has a pore size of 40μm and the second filter has a pore size of 10μm. The filters may be made of any materials although wire and nylon meshes are preferred.

The separated cells may be resuspended in appropriate nutrient media. The resuspension media may, for example include supplements such as serum. Preferably, the resuspension media includes glutamate supplementation and an agent to inhibit the overgrowth of dividing cells.

In a further preferred embodiment the support means is a solid substrate such as glass slide, a plastic slide, a glass tissue culture dish or a plastic tissue culture dish. Preferably,the support means is coated with one or more matrix substances selected from the group consisting of laminin, fibronectin and Matrigel. Preferably, the matrix substance is laminin.

Once the primary cell culture has been established by attachment to a solid support, the nutrient media may be decanted and replaced with a serum-free media. The cell culture is then maintained by feeding with serum-free media.

In a preferred embodiment the serum-free nutrient media includes a commercial media preparation with a valine supplement, such as MEM D-val [Gibco], and a serum free supplement, such as Monomed A (CSL).

In a second aspect the present invention provides a cell population which has been cultured by a method according to the first aspect of the present invention.

It will be appreciated that the cell culture system of the present invention provides a valuable tool in the study of isolated cells and in the study of neuronal-glial interactions which is not feasible in an explant culture of a piece of whole tissue. Furthermore, the cell populations are heterogeneous, as occurs in normal tissue, and therefore allow the study of processes which parallel normal behaviour. The present inventor has also found that neuronal cells cultured according to the present invention have extensive neurite outgrowth making them suitable for studies of axonal outgrowth and growth cones *in vitro*.

Further advantages of the present invention are that the cell culture system is maintained in serum-free conditions and that there are no exogenous growth factors required for maintenance. The cell culture system of the present invention therefore provides a means for testing the effectiveness of known neuronal and glial growth factors and also for screening novel growth factors. The system also provides a means for screening and measuring the effects of neurotoxins and potential neuroprotective agents. The system further provides a means for screening for any agent which affects the activity of cells. By "activity" we mean any activity of interest, examples of which include differentiation and intercellular communication. It will also be appreciated that the cell culture system of the present invention provides a means for screening for

therapeutic or prophylactic agents which may be used to treat or prevent a range of disease states. Such disease states may include neurodegenerative disorders such as Parkinson's Disease.

Accordingly, in a third aspect the present invention provides a method of screening an agent for neurotoxic or neuroprotective activity which method includes addition of the agent to a population of cells cultured by a method according to the first aspect of the present invention.

In a fourth aspect the present invention provides a method of screening an agent for activity which affects the growth of cells which method includes addition of the agent to a population of cells cultured by a method according to the first aspect of the present invention.

In a fifth aspect the present invention provides a method of screening for an agent which affects the activity of cells which method includes adding a putative agent to a population of cells cultured by a method according to the first aspect of the present invention and monitoring the activity of the cell population.

In a sixth aspect the present invention provides a method of screening for a therapeutic or prophylactic agent which method includes addition of a putative therapeutic or prophylactic agent to a population of cells cultured by a method according to the first aspect of the present invention.

The present inventor has detected (by RT-PCR) mRNAs for a variety of odorant receptors in olfactory neurons cultured by the method of the present invention. This observation confirms that cells cultured by the present method have the genetic machinery to express odorant receptor proteins. Accordingly, the culture method of the present invention is also potentially useful for studies of odorant receptor function. As will be understood by those of skill in the art, odorants are compounds which have the ability to stimulate neuronal cells above their basal level of excitement. The culture method of the present invention may therefore be used to screen for odorants and antagonists of oderants. Antagonism may be readily determined by exposing the cultures of the present invention to the odorant alone, and to the odorant in admixture with a potential antagonist. A decrease in the level of excitation in the presence of the putative antagonist relative to the control levels in the presence of the odorant alone, identifies an antagonist.

The culture method of the present invention may also be useful for producing cells for transplantations or for nerve repair operations or acute therapies for spinal cord injury. For example, the cultured cells may be induced to express a neurotrophic factor, such as GDNF, which has been shown to play an important role in olfactory neurons *[Buckland, M., Pemper, F & Cunningham, A. (1998) GDNF: A Complex Trophic Role in the Olfactory System. St Vincents Hospital Symposium, Sept, Sydney; Buckland, M. & Cunningham, A. (1997) Changes in Expression of Neurotrophic Factors in the Regenerating Olfactory Neuroepithelium. Proc. Aust. Neuroscience Soc., 8: Cunningham, A.M. & Buckland, M. (1997) Alterations in the Neurotrophic Factors BDNF, GDNF and CNTF in the Regenerating Olfactory System. ISOT XII & AChemS XIX, San Diego, July.]* An implant of induced cultured cells infiltrating, for example, a semipermanent dissolvable collagen mesh could be overlaid on damaged tissues (for example, transected spinal cord or the basal ganglia in Parkinson's disease) providing an ongoing (eg. 1-2 weeks) *in vivo* source of neurotrophic factor. It may be possible to take primary cells from a patient, culture the cells according to the method of the present invention in order to induce expression of a neurotrophic factor, such as GDNF, then implant the cells back into the patient. In this case the transplant would be autologous and would therefore avoid problems of rejection.

DETAILED DESCRIPTION OF THE INVENTION

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following example.

**Example 1**

Method of cell culture:

Dissect olfactory tissue from 45 x 0-2 day old rat pups and place into 10cm petri dish with 15 mls of DMEM [Gibco] + 0.9% hepes [Sigma], this is designated MEM-air. Place tissue and DMEM-air into a 50ml centrifuge tube, rinse petri dish with 15 mls of MEM-air then spin down tissue pieces for 10 mins at 500 rpm. While cells are spinning add DMEM-air to BSA (Sigma) 10 mg/ml, dispase 11 (Boehringer Mannheim) 6.2 mg/ml, collagenase D (Boehringer Mannheim) 1.12 mg/ml, hyaluronidase (Sigma) 0.9 mg/ml, Dnase 1 (Sigma) a sprinkle of powder. Filter through 0.45 μm and 0.22 μm filters. Aspirate off supernatant leaving about 200ml of DMEM-air. Place

tissue pieces into 10cm petri dish. Mince tissue with sterile scissors for 5 mins then add filtered enzymes. Add tissue to a sterile conical flask containing a small stirring bar and incubate solution for 60 mins at 37oC in a water bath. Wet a wire mesh [150 µm, Small Parts, Inc] with DMEM-air and filter digested tissue using a cell scraper to push the tissue through. Place cells into a 50ml centrifuge tube and spin for 10 mins at 750 rpm. Aspirate off enzyme solution and tap tube gently on bench to dislodge pellet. Add 10 mls of serum containing media (MEM D-val (Gibco) containing 10% foetal bovine serum, 2mM glutamate, 2.5mg/ml fungizone, Kanamycin monosulfate 100mg/L, Gentamicin sulfate 50mg/L, 10uM cytosine arabinoside and Monomed A(CSL)),and triturate gently until a single cell suspension is obtained. Add another 10 mls of serum containing media, mix then pass suspension through a 40 µm cell strainer (Falcon), then a 10 µm mesh filter (Small Parts Inc.). Plate cells at $6 \times 10^5$ cells per ml onto glass chamber slides [Nunc] previously coated overnight at 37°C with 10mg/ml laminin (Integrated Sciences). After 24 hours change media to serum free media which consists of MEM D-val, [Gibco], 2mM glutamate, 2.5mg/ml fungizone, [Trace], 10uM cytosine arabinoside, [Sigma] gentamycin/kanamycin, and Monomed A, [Trace]. Cells are then fed 42 hourly and survive for up to three weeks in culture.

**Example 2**

The neurons, glia and progenitor cells in culture according to the present invention have been further characterised using immunocytochemical markers.

The neurons have been shown to express β-tubulin isotype 3, GAP43 and NCAM (neural cell adhesion molecule). This confirms their neuronal phenotype and provides evidence that they retain some immature characteristics rather than being fully terminally differentiated, mature neurons. They also do not express at significant levels OMP (olfactory marker protein) immunoreactivity. OMP is the classic marker of fully differentiated mature olfactory neurons so this provides further confirmation of their immature status.

The glial cells in culture have been shown to be significantly heterogeneous with the majority of cells expressing S-100 marker protein and the low affinity nerve growth factor receptor which is consistent with them being of the ensheathing cell, peripheral glial phenotype. Some of the glial

cells express GFAP (glial fibrillary acidic protein) at high levels and are in keeping morphologically with central, astrocytic glial cells. The cells of the peripheral glial phenotype also express GFAP but at much lower levels.

The progenitor cells in culture have been identified by their morphology and intense immunoreactivity for cytokeratin.

Hence we have confirmed the heterogeneity of cells in culture which reflects the diversity of cells in the source tissue. The immaturity of the olfactory neurons may be the reason for the plasticity of the cells, their capacity for longterm survival *in vitro* ie up to 3 weeks, and potentially may make them more promiscuous and useful for trophic factor work.

**Example 3**

Cells cultured by the method of the present invention have been used to assay responsiveness of the classes of cells to neurotrophic factors. The recombinant factors studied include: nerve growth factor (NGF), glial cell line derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), brain derived neurotrophic factor (BDNF) and leukemia inhibitory factor (LIF). NGF, GDNF, CTNF and BDNF were all negative for effects on neurons in culture when assayed for effects on neuronal cell number, phenotype and immunocytochemistry for markers of differentiation. LIF, in preliminary experiments, has been shown to increase the numbers of neuronal cells at day 5 in culture.

The recombinant growth factors assayed were from Collaborative Research (NGF), Promega (GDNF, CNTF and BDNF) and Amrad (Esgrow, LIF). For NGF, GDNF, CNTF and BDNF, cells were plated as described in the method and the recombinant factors to be assayed added at the first change of media and feed which occurred at 24 hours in culture. Cells were then examined at 5 and 7 days in culture. Assays included an overall cell density count on unfixed living cells; and phenotypic analysis and counts of healthy neurons and glia using the immunocytochemical markers B-specific tubulin (neurons), GFAP (glia), cytokeratin (progenitor cells) as well as the marker of olfactory neuronal differentiation, OMP. Preliminary assays showed no significant change from control cultures in the number of cells in the neuronal or glial class or in the number of differentiated neurons.

For LIF, initial results suggest there is a definite increase in viable, B-specific tubulin immunoreactive cells at 5 days in culture. In future experiments, to explore the mechanism of the effect, we will perform

tritiated thymidine assays or bromodeoxyuridine incorporation assays to determine if LIF causes increased neurogenesis from progenitor cells. An alternate mechanism is that LIF may promote the survival of neurons already in culture.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

CLAIMS:

1. A method of culturing a mixed population of neuronal and glial cells which method includes

(i) providing a tissue sample which includes a heterogeneous population of neuronal and glial cells;

(ii) dissociating the neuronal and glial cells by enzymatic digestion;

(iii) separating the dissociated neuronal and glial cells from aggregate cells;

(iv) plating the dissociated cells on a support means until the cells have attached thereto and grown to a desired concentration; and

(v) maintaining the cell population attached to the support by addition of serum-free media.

2. A method according to claim 1 wherein the tissue sample is olfactory neuroepithelial tissue.

3. A method according to claim 1 or claim 2 wherein the tissue is derived from a rat, mouse, primate or human.

4. A method according to any one of claims 1 to 3 wherein the tissue is derived from a neonatal rat.

5. A method according to any one of claims 1 to 4 wherein the tissue is minced to provide small pieces of tissue prior to the step of enzymatic dissociation.

6. A method according to any one of claims 1 to 5 wherein the enzymatic digestion involves the use of one of more enzymes selected from the group consisting of dispase, collagenase, hyaluronidase and DNAse.

7. A method according to any one of claims 1 to 6 wherein the dissociation of neuronal and glial cells further includes mechanical dissociation.

8. A method according to claim 7 wherein the mechanical dissociation involves trituration.

9. A method according to any one of claims 1 to 8 wherein the separation of the dissociated cells involves selective filtering of neuronal and glial cells.

10. A method according to claim 9 wherein the filtering includes passing the cells through a first filter which has a pore size of from about 40 to 60 μm and a second filter which has a pore size of from about 8 to 15 μm.

11. A method according to claim 10 wherein the first filter has a pore size of about 40μm.

12. A method according to claim 10 or claim 11 wherein the second filter has a pore size of about 10μm.

13. A method according to any one of claims 10 to 12 wherein the first and/or second filter is selected from a wire mesh and a nylon mesh.

14. A method according to any one of claims 1 to 13 wherein the support means is selected from a glass slide, a plastic slide, a glass tissue culture dish or a plastic tissue culture dish.

15. A method according to any one of claims 1 to 14 wherein the support means is coated with one or more matrix substances selected from the group consisting of laminin, fibronectin and Matrigel.

16. A method according to claim 15 wherein the matrix substance is laminin.

17. A cell population cultured by a method according to any one of claims 1 to 16.

18. A method of screening an agent for neurotoxic or neuroprotective activity which method includes addition of the agent to a cell population according to claim 17.

19. A method of screening an agent for activity which affects the growth of cells which method includes addition of the agent to a population of cells according to claim 17.

20. A method of screening for an agent which affects the activity of cells which method includes adding a putative agent to a population of cells according to claim 17 and monitoring the activity of the cell population.

21. A method of screening for a therapeutic or prophylactic agent which method includes addition of a putative therapeutic or prophylactic agent to a population of cells according to claim 17.

# INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00616

**A. CLASSIFICATION OF SUBJECT MATTER**

Int Cl[6]: C12N - 005/02, 06, 08, 00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
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**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | Glia 1, pages 380-392 (1988) Tropea, M. et al "Glial Cells Promote Dendritic Development in Rat Sympathetic Neurons In Vitro." See page 383 column 2 | 1-21 |
| X | Neuron 8, pages 1191-1204 (1992) Pixley, S.K. "CNJS Glial Cells Support In Vitro Survival, Division and Differentiation of Dissociated Olfactony Neuronal Progenitor Cells". see page 1192 column 2 paragraph 3 - page 1196 and Discussion. | 1-21 |

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

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International Application No.
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C (Continuation) **DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | J. Basic Clin. Physiol. Pharmacol. 4(3) pages 157-179 (1993) Hanani, M. "Neuron and Glial Cells of the Enteric Nervous System : Studies in Tissue Culture". see sections 1.3, 2.1.2, 2.1.6. and 3.1 | 1-21 |
| X | J. Neurosci. Res. 41 pages 246-258 (1995) Martin, F.C. and Wiley, C.A. "A Serum-Free, Pyruvate-Free Medium that Supports Neonatal Neural/Glial Cultures". see Materials and Methods and Discussion | 1-21 |
| X | Exp. Neurology 138 pages 144-157 (1996) Nakao, N. et al, "Differential Trophic Effects of Basic Fibroblast Growth Factor, Insulin-Like Growth Factor-1 and Neurotrophin-3 on Striatal Neurons in Culture". see pages 150-152 | 1-21 |

# INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00616

**Box B continued**

Electronic data base continued

MEDLINE : (CULTURE MEDIA, SERUM FREE/CT) AND (CULTURE (5N) GLIA#) AND (CULTURE (5N) (NEURON OR NEURONAL)) AND (1992-1997)
CHEM ABS :(CELLS, CULTURED/CT) AND (CULTUR? (5N) (NEURON OR NEURONAL)) AND (CULTURE (5N) GLIA?) AND (1992-1997)